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## Three-dimensional Structure and Disulfide Bond Connections in Bovine Pancreatic Phospholipase A<sub>2</sub>

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AND PHILIP J. VANDERMAELEN

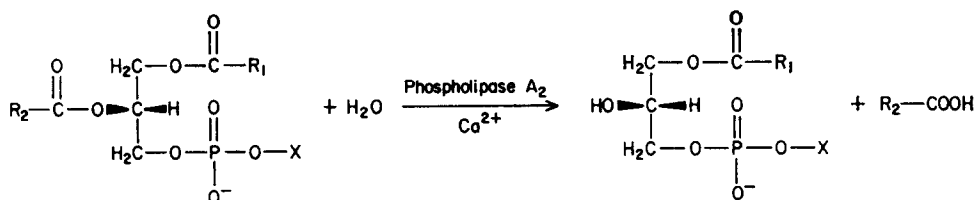
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Bovine pancreatic phospholipase A<sub>2</sub> ( $M_r = 14,000$ ) has been crystallized and its three-dimensional structure determined by X-ray diffraction analysis to a resolution of 2.4 Å. Three heavy-atom derivatives were used in the phase calculations with inclusion of the anomalous dispersion differences. The resulting electron density map allowed an easy and unambiguous tracing of the peptide chain. Two of the seven disulfide connections appeared to be different from what was suggested by the earlier chemical and structural work. The bovine phospholipase A<sub>2</sub> structure contains about 50%  $\alpha$ -helix and 10%  $\beta$ -structure. The bovine enzyme structure was found to deviate substantially from the previously published porcine phospholipase structure.

### 1. Introduction

Phospholipase A<sub>2</sub> catalyses specifically the hydrolysis of the ester bond at the C<sub>2</sub> position of 3-*sn*-phosphoglycerides:



The group X represents any of the naturally occurring residues found in phosphoglycerides. Calcium is an essential cofactor for this reaction. The enzyme has been isolated from mammalian pancreatic tissue (De Haas *et al.*, 1968; Dutilh *et al.*, 1975), and from snake (Wells & Hanahan, 1969) and bee venom (Shipolini *et al.*, 1971). In micro-organisms and in various mammalian tissues an intracellular phospholipase activity has been demonstrated (see e.g. Brockerhoff & Jensen, 1974). The pancreatic enzyme occurs as a zymogen in the pancreatic juice. After secretion in the gastrinal tract, the enzyme is activated by trypsin, which removes seven amino acid residues from the N-terminus. Whereas the zymogen can degrade monomeric substrate molecules, the activated enzyme is, in addition, able to digest, very efficiently, ordered phospholipid structures like micelles. The bovine active enzyme has a molecular

weight of 14,000. Its peptide chain consists of 123 amino acid residues and the sequence shows a great deal of homology with other phospholipases A<sub>2</sub> (Fleer *et al.*, 1978; Strydom, 1977). The enzyme has 14 cysteine residues in seven disulfide bridges and no free sulfhydryl groups. In the porcine enzyme the connections between five of the seven pairs of cysteines have been determined, the connections between the remaining four cysteines being tentatively assigned (De Haas *et al.*, 1970; Puyk *et al.*, 1977). To obtain a fuller understanding of protein-lipid interactions and the reaction mechanism of phospholipid degradation we undertook an X-ray structure determination of pancreatic phospholipases A<sub>2</sub>. Initial studies concerned the *porcine* enzyme. Crystals of the active form of the porcine enzyme could not be obtained, but it was possible to grow crystals, albeit of poor quality, of its zymogen. The solvent content of these crystals was rather high (62%) and the diffraction pattern did not extend to a resolution beyond 3 Å. A single heavy-atom derivative (K<sub>3</sub>UO<sub>2</sub>F<sub>5</sub>) was used for the structure determination, with inclusion of the anomalous scattering data of the uranyl atom (the SIRAS method). An electron density map of the *porcine* proenzyme was calculated to a resolution of 3 Å (Drenth *et al.*, 1976). The interpretation of this rather poor map was supported by biochemical data and the sequence information available at that time. The molecule had the shape of a rectangular box with dimensions of approximately 25 Å × 28 Å × 35 Å. It contained neither regular  $\alpha$ -helices nor  $\beta$ -pleated sheets.

However, when a revised porcine phospholipase A<sub>2</sub> sequence became available (Puyk *et al.*, 1977) it was, unfortunately, not possible to incorporate all modifications into the structure.

By that time we had observed that the enzyme and proenzyme from bovine pancreas crystallized readily as high quality crystals.

This prompted us to initiate an independent high-resolution structure determination of these enzymes rather than to reinterpret the porcine phospholipase electron density map.

We report here the three-dimensional structure of the active form of phospholipase A<sub>2</sub> from *bovine* pancreas. It was found to deviate substantially from the previously published *porcine* proenzyme structure.

## 2. Experimental Procedures

Crystallisation was accomplished in the following way: freeze-dried protein was dissolved in Tris buffer (100 mM-Tris, 5 mM-CaCl<sub>2</sub>, pH 7.6) to a final concentration of 3% (30 mg protein/ml buffer): 50  $\mu$ l of the protein solution was frozen at -18°C; 50  $\mu$ l of 2-methyl-2, 4-pentanediol (Fluka) was layered on top of the frozen protein solution. After 1 to 2 weeks at room temperature (20°C), crystals had grown with sizes up to 0.5 mm × 0.5 mm × 0.8 mm. The crystals had the orthorhombic spacegroup *P*2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>, with cell dimensions  $a = 47.07$  Å,  $b = 64.45$  Å and  $c = 38.15$  Å. The asymmetric unit contained 1 molecule; the solvent content was 39%. The diffraction pattern extends to at least 1.8 Å. The crystals, being not very susceptible to radiation damage, could be exposed for up to 120 to 150 hours, in the beam of a 1.5 kW sealed-off X-ray tube with copper anode, before intensities of reference reflections had dropped more than 15%.

Intensities of native protein crystals and 3 heavy-atom derivatives were measured on an ENRAF-NONIUS CAD4 diffractometer to a resolution of 2.4 Å. The initial heavy-atom parameters were obtained from difference Pattersons. These parameters were refined using alternate cycles of phase calculation (Blow & Crick, 1959) and lack-of-closure error refinement (Dickerson *et al.*, 1961). Anomalous dispersion differences were used in the phase calculations (North, 1965). Isotropic temperature factors were applied. Table 1 summarizes some results of the refinement of heavy-atom parameters. As can be judged

TABLE 1

*Results of the refinement of heavy-atom parameters at 2.4 Å resolution*

Derivative	Number of positions	Number of crystals used	$R_c$ †	$f_{\text{calc}}/ \langle E_{\text{centric}} \rangle$
K <sub>2</sub> Pt(CN) <sub>4</sub>	2	4	0.369	2.04
Cd(NO <sub>3</sub> ) <sub>2</sub>	2	6	0.469	1.58
K <sub>2</sub> PtI <sub>6</sub> ‡	3	6	0.320–0.492	1.70–1.21

$$\dagger R_c = \frac{\sum |F_{\text{PHobs}}| - |F_{\text{PHcalc}}|}{\sum |F_{\text{PHobs}} - F_{\text{P}}|} \text{ for centric reflections.}$$

‡ Due to a variable occupancy of the heavy-atom binding sites, the data from individual crystals were refined separately.

from this Table, the K<sub>2</sub>Pt(CN)<sub>4</sub> derivative is of excellent quality, the other two still being suitable for inclusion in the phase calculations: 4039 centroid phases with an overall figure of merit of 0.802 were used for calculating an electron density map to a resolution of 2.4 Å. For interpretation the map was drawn on a scale of 1.75 Å/cm. Preliminary co-ordinates of C $\alpha$  and S $\gamma$  were determined from this map.

### 3. Description of the Structure

The electron density map allowed an easy tracing of the polypeptide chain. Most of the side-chains were clearly visible. The disulfide bonds, being the highest peaks in the electron density, showed up unmistakably. The phenyl ring of tyrosine 25

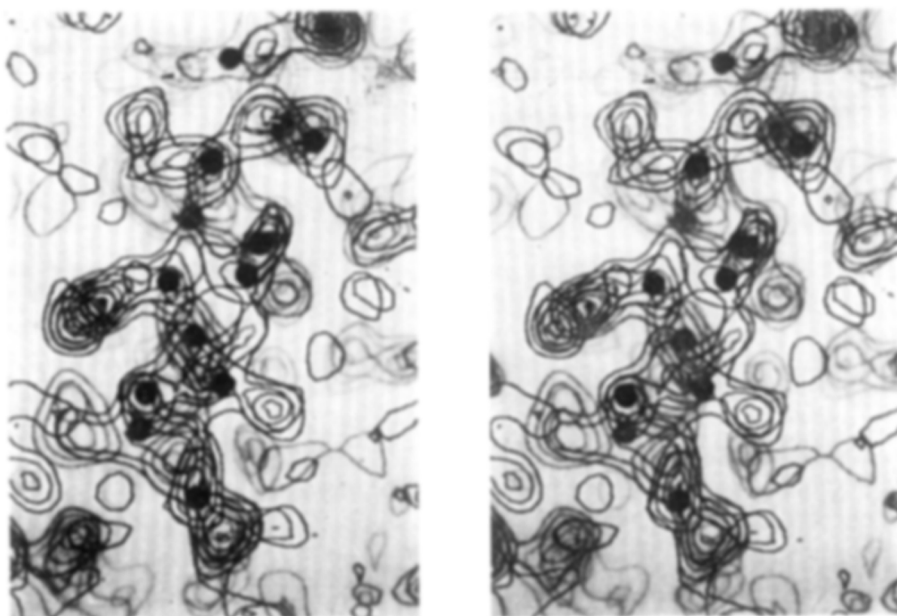


FIG. 1. Part of the electron density map showing the first residues of  $\alpha$ -helix C. In this part of the molecule the amino acid side-chains are among the most conserved residues in all sequences known up to now. The black filled circles indicate the approximate position of the  $\alpha$ -carbon atoms. The sequence shown is, from bottom to top: Asp40-Leu41-Asp42-Arg43-Cys44-Cys45-Gln46-Thr47-His48-Asp49-Asn50-Cys51-Tyr52-Lys53.

shows a distinct depression in the center of its ring. A portion of the electron density map is shown in Figure 1. The molecule has the shape of a flattened cylinder with dimensions of approximately  $22 \text{ \AA} \times 30 \text{ \AA} \times 42 \text{ \AA}$  (Figs 2 and 3). The most conspicuous features are the high content of secondary structure and the disulfide bond connections, which very tightly hold together the various stretches of  $\alpha$ -helix and  $\beta$ -structure (Fig. 4).

The backbone of the molecule consists of two long antiparallel  $\alpha$ -helices (helices C and E in Fig. 4, corresponding with amino acid numbers 40 to 58 and 90 to 108), each having five full turns. These two helices are connected by two disulfide bonds: Cys44—Cys105 and Cys51—Cys98, which diminishes the mobility of these two helices with respect to each other. This might be important for the position of various active site residues, which are part of these two helices. The short helix D (residues 59 to 64) makes an angle of about  $75^\circ$  with helix C. This short helix is also linked by a disulfide bond to helix E (Cys61—Cys91). The connecting polypeptide between leucine 64 and alanine 90 has no obvious secondary structure, except for residues 74 to 78 and 80 to 85, where the chain forms two antiparallel  $\beta$ -strands. One of these  $\beta$ -strands is covalently linked to  $\alpha$ -helix E through Cys84—Cys96. The other is connected by a disulfide bond to the N-terminal region: Cys77—Cys11. This N-terminal region is also in an  $\alpha$ -helical conformation (helix A, residues 1 to 13) with the terminal  $\text{NH}_2$  of alanine 1 pointing into the active site. From residue 14 to 40 the peptide chain is in a random coil, except for residues 19 to 22, which possibly form one  $\alpha$ -helix turn (helix B) at the surface of the molecule. In this region the side-chains are somewhat diffuse in the electron density map. This section of the polypeptide chain is linked by a disulfide bridge to helix C (through Cys29—Cys45). The last part of the peptide chain (residues 109 to 123) forms a long surface loop with the C-terminal cysteine 123 connected to cysteine 27 by a disulfide bond. Altogether about 50% of the amino acid residues are in an  $\alpha$ -helix conformation, and about 10% in  $\beta$ -structure.

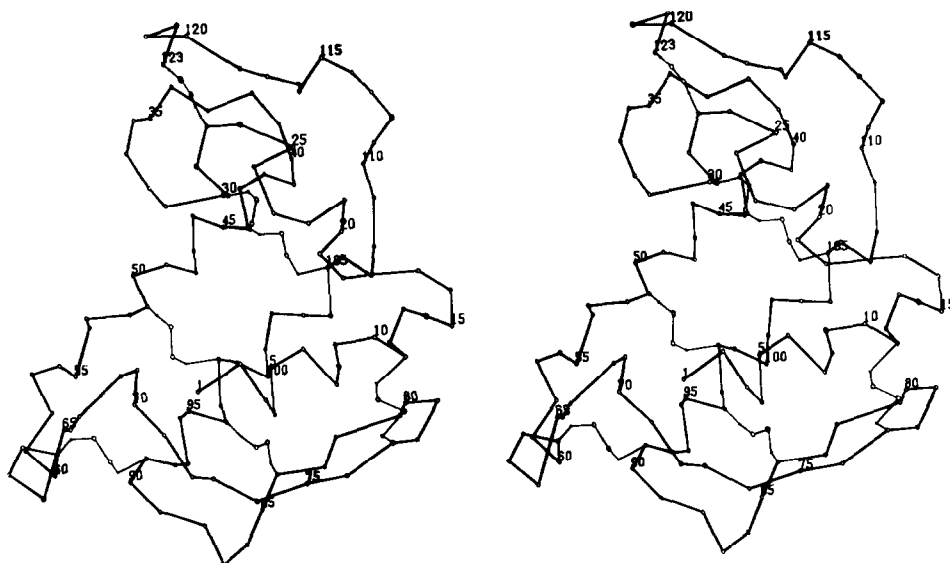


FIG. 2. Stereo diagram showing the conformation and disulfide bridges of the bovine pancreatic phospholipase molecule.

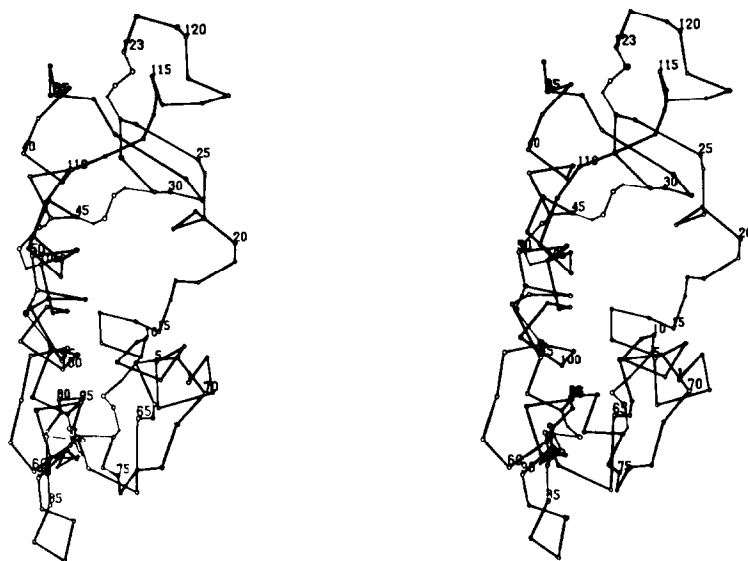


FIG. 3. Stereo diagram showing C $\alpha$ -carbon atoms. The phospholipase molecule is rotated by 90° about the vertical axis with respect to Fig. 2.

It is easy to accommodate the sequence of homologous phospholipases into the bovine phospholipase A<sub>2</sub> structure. The position of insertions and deletions is always at the molecular surface.

Histidine 48 is reported to be part of the active site of phospholipase A<sub>2</sub>; acylation of this residue destroys enzymatic activity (Volwerk *et al.*, 1974). As can be seen from Figure 2, this histidine residue is in a cavity at the molecular surface. From the upper side of this cavity aspartic acid 49 is directed towards the histidine, whereas aspartic acid 99 points up from below into the cavity. Near this latter side-chain, in the

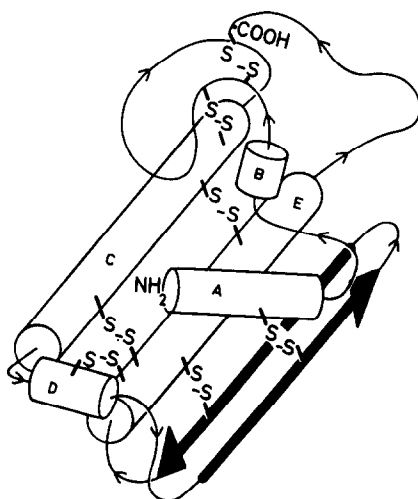


FIG. 4. Schematic drawing showing the helices and  $\beta$ -structure of the phospholipase molecule.

vicinity of the histidine, is some extra electron density, which can be interpreted as a calcium ion. The hydroxyl group of tyrosine 52 is in contact with this density.

#### 4. Discussion

In contrast to the sequence numbering scheme used for the porcine phospholipase structure, we have adapted here the numbering of the active horse phospholipase (Evenberg *et al.*, 1977) for reasons given in that paper. The same numbering scheme was used for the revised porcine enzyme (Puyk *et al.*, 1977) and the bovine phospholipase sequences (Fleer *et al.*, 1978).

As is obvious from the electron density map, the disulfide bridge connections used until now have to be revised. The correct disulfide connections are listed in Table 2. Chemically determined connections (De Haas *et al.*, 1970) are indicated.

TABLE 2  
*Correct disulfide connections*

Chemically determined	
(1) Cys11—Cys77	Yes
(2) Cys27—Cys123	Yes
(3) Cys29—Cys45	Yes
(4) Cys44—Cys105	No
(5) Cys51—Cys98	No
(6) Cys61—Cys91	Yes
(7) Cys84—Cys96	Yes

For the interpretation of the electron density map of *porcine* phospholipase we had to rely heavily on the sequence and the proposed disulfide bond connectivity. In the porcine enzyme a disulfide bridge between cysteine 51 and cysteine 105 was tentatively assigned. We now know that this is not correct. During the building of the porcine proenzyme model some revisions of the porcine sequence became available, including two extra cysteine residues (Cys44 and Cys98). Having incorporated these cysteine residues into the structure, it appeared that their side-chains were sufficiently close for a disulfide bond connection. Moreover, this bond coincided with a high electron density peak. Nevertheless, the interpretation of the porcine map in this region is incorrect. In the porcine structure the polypeptide chain from residues 44 to 51 was interpreted as running parallel to the polypeptide chain 98 to 105. From the bovine electron density map it is clear that these stretches run in opposite directions and that the disulfide connections are as listed in Table 2. This implicates further changes in the interpretation in the porcine map. Another difference between the active bovine enzyme and the porcine proenzyme is the high content of secondary structure in the bovine enzyme compared with hardly any in the porcine proenzyme. Whether this difference in secondary structure is real or not will be discussed after completion of the bovine proenzyme structure determination at high resolution, which is in progress in this laboratory. However, we should mention in this respect that from circular dichroism studies (Seanu *et al.*, 1969; Jirgensons & De Haas, 1977), a high helix content (50 to 55%) could be predicted for these enzymes.

Two or more long antiparallel  $\alpha$ -helices have been observed in other proteins of similar size (e.g. the apoferritin subunit; Banyard *et al.*, 1978). However, the large extent of disulfide connections as found between the bovine phospholipase helices does not occur frequently. It accounts for the enzyme's stability against denaturing reagents and conditions. The adaptive purpose of such a structure is possibly the protection of the enzyme against denaturation at the phospholipid-water interface, which is its natural place of activity (Brockerhoff & Jensen, 1974). Calcium is an essential cofactor for phospholipase activity both for the zymogen and the active enzyme. From the ultraviolet difference spectrum of porcine phospholipase A<sub>2</sub> with or without calcium it was concluded that, upon binding of calcium a tyrosine side-chain becomes partially shielded from the aqueous solvent (Pieterse *et al.*, 1974). Furthermore, it was tentatively concluded that binding of calcium induced a charge effect on a histidyl residue. We interpreted an electron density peak near histidine 48, tyrosine 52 and aspartic acid 99 as a calcium ion. As these three residues invariably occur at equivalent positions in all phospholipases A<sub>2</sub> with known sequences (except for bee venom phospholipase, where alignment with the sequences of other phospholipases is not yet unambiguous), our proposed position of the calcium ion seems reasonable. Further details of the active site will be published later.

The high activity of the active enzyme towards micellar substrate cannot yet be explained. However, an interesting feature is the terminal NH<sub>2</sub> group of alanine 1 in the active site. It is located within hydrogen bond distance from what seems to be a water molecule, which possibly is liganded with the active site calcium ion. The terminal NH<sub>2</sub> group does not form a salt bridge with a carboxylate as suggested previously (Abita *et al.*, 1972; Van Dam-Mieras *et al.*, 1975).

In the zymogen an extra heptapeptide is bound to alanine 1 and the alanine 1 NH<sub>2</sub> group is blocked. This might cause conformational differences in the active site region between the active enzyme and its zymogen.

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